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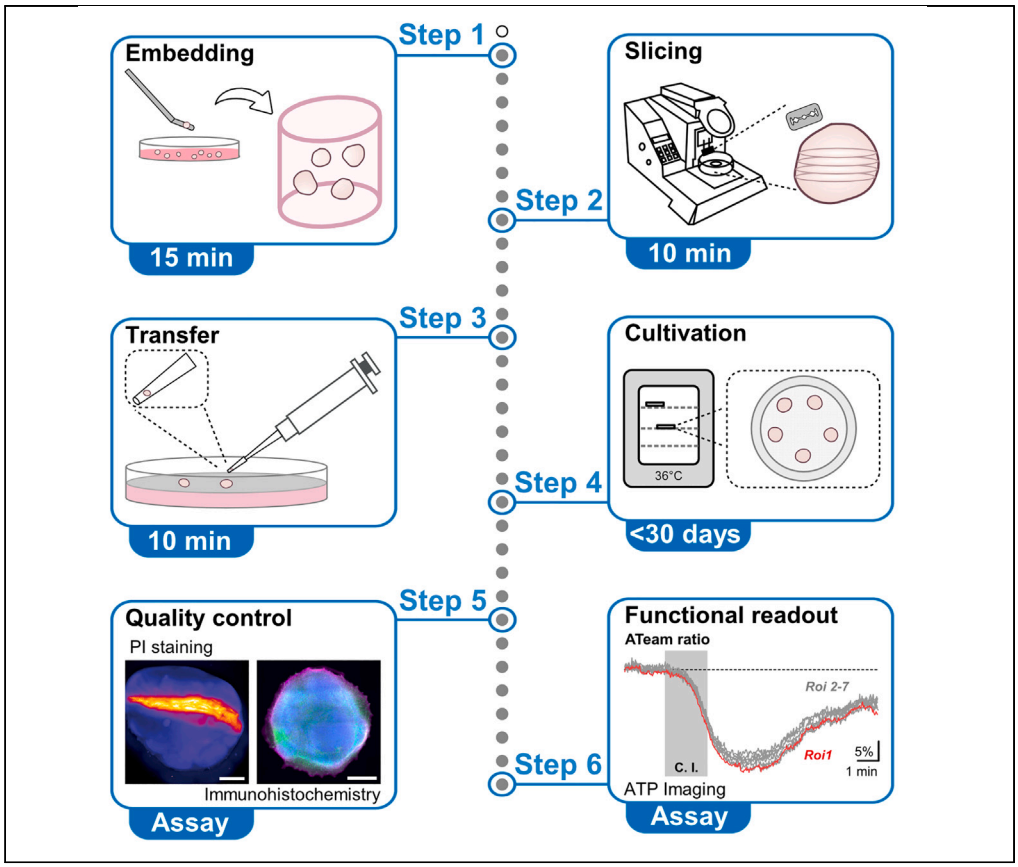
Protocol for the generation of cultured cortical brain organoid slices



Three-dimensional brain organoids from human pluripotent stem cells are a powerful tool for studying human neural networks. Here, we present a protocol for generating cortical brain organoid slices (cBOS) derived from regionalized cortical organoids and grown at the air-liquid interphase. We provide steps for slicing organoids and maintaining them in long-term culture. We then detail approaches for quality control including the evaluation of cell death and cellular identity. Finally, we describe procedures for the expression of a genetically encoded nanosensor for ATP.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to prepare robust human cortical brain organoid slices (cBOS)

Detailed steps for maintenance of cBOS in long-term culture

Evaluation of cell death and identification of different cell types in cBOS

Functional characterization of cells using fluorescence-based nanosensors

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Protocol for the generation of cultured cortical brain organoid slices

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SUMMARY

Three-dimensional brain organoids from human pluripotent stem cells are a powerful tool for studying human neural networks. Here, we present a protocol for generating cortical brain organoid slices (cBOS) derived from regionalized cortical organoids and grown at the air-liquid interphase. We provide steps for slicing organoids and maintaining them in long-term culture. We then detail approaches for quality control including the evaluation of cell death and cellular identity. Finally, we describe procedures for the expression of a genetically encoded nanosensor for ATP.

For complete details on the use and execution of this protocol, please refer to Petersilie et al.[1]

BEFORE YOU BEGIN

In the present paper, we describe a protocol which is based on regionalized cortical organoids. These appear to be less heterogeneous and more robust than whole-brain self-patterned cerebral organoids[2,3] and thus represent an ideal starting point for the generation of uniform cultured brain organoid slices (cBOS).[1,4] cBOS can be employed as a platform for probing cellular function and/or dysfunction using a variety of approaches including electrophysiology or calcium imaging.[1] Moreover, cBOS allow the expression of genetically-encoded sensors and probes using suitable viral vectors. Altogether, cBOS provide a strategy widely applicable to different laboratories for enabling functional assessment of developing human neural networks.

Preparation of cortical organoids

Timing: 0–79 days

Three-dimensional (3D) brain organoids from human pluripotent stem cells (hPSCs) represent a powerful tool to study human neurodevelopment. Functional probing of neural cells and networks within these sophisticated 3D structures can, however, be cumbersome. Moreover, brain organoids may develop necrotic cores upon long-term culturing. These problems are largely circumvented by preparation of slices from brain organoids for long-term culture on the air-liquid interphase, a procedure which has been introduced initially by the Lancaster laboratory.[5–8]

1. Prepare regionalized cortical organoids according to the published procedures described in Le et al. (2021)[9] and Petersilie et al. (2024).[1]

Note: Cortical organoids prepared according to these protocols display a spherical and uniform morphology, which is the basis for the preparation of slices with a uniform roundish appearance.

Preparation for embedding of organoids

Timing: 2 h

2. Prepare a custom-built mold (cut upper end of a 10 mL syringe, inner diameter ~1.5 cm) or use a commercial mold (22 × 22 × 20 mm).
3. Place the following items on your embedding (sterility not mandatory, but advantageous) workstation.
   a. Heating block.
   b. Ice box.
   c. Hank's balanced salt solution (HBSS).
   d. Absorbent filter paper.
   e. Filter paper.
   f. Pasteur pipettes (3 mL).
   g. Spatula.
4. Prepare a 3% low melting point (LMP) agarose mixture (solubilized in HBSS, in non-sterile conditions).
   a. Pre-heat LMP agarose (90°C) and slowly cool it down to 40°C before use.

Preparation for slicing of organoids

Timing: 10 min

5. Insert a new and clean blade in vibratome.
6. Store cooling element and bath at −20°C.
7. Store HBSS at 4°C.
8. Place the following items on your slicing workstation.
   a. Glass Pasteur pipette.
   b. Glue.
   c. Mounting plate.

Preparation for transfer of organoid slices onto membranes

Timing: 10 min

9. Place Petri dishes filled with HBSS in the incubator (36°C and 5% CO_2).
10. Prepare a six-well plate filled with 750 µL media (here: Cortical Differentiation Medium IV (CDM IV) or Neural Differentiation Medium (NDM)) and culture plate inserts and put it into the incubator (36°C and 5% CO_2).

Note: Four Petri dishes are needed per agarose block.

11. Place sterile glass Pasteur pipettes in laminar flow cabinet.

Note: Four pipettes are needed per agarose block.

Institutional permissions

Experiments must be performed in accordance with all relevant institutional and national guidelines and regulations. Before starting, permissions and approvals must be acquired from the relevant institutions to ensure that experiments are in compliance with regulatory guidelines that are in effect for induced pluripotent stem cells (iPSCs) derived from humans. In this study, ethical approval for using control human iPSCs in a pseudo-anonymized fashion was obtained from the Ethic Committee of the University Clinic Düsseldorf (study number 2019–681 approved on October 11, 2019).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strain		
ssAAV-2/2-hSyn1-ATeam1.03YEMK-WPREhGHp(A)	ETH Zürich	v244
Chemicals, peptides, and recombinant proteins		
(+)-Sodium L-ascorbate	Sigma-Aldrich	Cat#A4034; CAS: 134-03-2
B-27 supplement (50x)	Thermo Fisher Scientific (Gibco)	Cat#17504044
B-27 supplement (50x) minus Vitamin A	Thermo Fisher Scientific (Gibco)	Cat#12587001
Chemically defined lipid concentrate	Thermo Fisher Scientific (Gibco)	Cat#11905031
cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA)	Sigma-Aldrich	Cat#D2534-25MG; CAS: 6217-54-5
Dibutyryl cAMP	STEMCELL Technologies	Cat#73886; CAS: 362-74-3
DMEM/F12, HEPES	Thermo Fisher Scientific (Gibco)	Cat#31330038
Fetal bovine serum	Thermo Fisher Scientific (Gibco)	Cat#10270106; CAS: 9014-81-7
GlutaMAX supplement	Thermo Fisher Scientific (Gibco)	Cat#35050061
Hank's balanced salt solution	Sigma-Aldrich	Cat#H9394
Heparin	Sigma-Aldrich	Cat#H3149-25KU; CAS: 9045-22-1
Human BDNF	MACS Miltenyi	Cat#130-096-286; CAS: 218441-99-7
MycoZap Plus-CL	Lonza	Cat#VZA-2012
N-2 supplement (100x)	Thermo Fisher Scientific (Gibco)	Cat#17502048; CAS: 10102-18-8
Neurobasal-A medium	Thermo Fisher Scientific (Gibco)	108880-22
Penicilin-Streptomycin (10.000 U/mL)	Thermo Fisher Scientific (Gibco)	Cat#15140122
Recombinant human EGF protein, CF	R&D Systems	Cat#236-EG-200; CAS: 62253-63-8
Recombinant human FGF basic/FGF2/bFGF (145 aa) protein, CF	R&D Systems	Cat#3718-FB-100; CAS: 106096-93-9
Recombinant human NT-3	PeproTech	Cat# PPT-450-03-50; CAS: 130939-66-1
Low melting point agarose; Alternative: Agarose, low gelling temperature	Thermo Fisher Scientific (Gibco); Sigma-Aldrich	Cat#55174B; CAS: 9012-36-6; Cat#A9413; CAS: 39346-81-1
Deposited data		
Raw and analyzed data	This paper	https://researchdata.hhu.de/handle/123456789/136
Experimental models: cell lines		
Human: HMGUi001-A (XM001) iPSC	Helmholtz Zentrum München (HMGU)	RRID: CVCL_WJ49, https://hpscreg.eu/cellline/HMGUi001-A; PMID: 29396371
Human: HHUUKDi009-A (TFBJ) iPSC	Heinrich Heine University Düsseldorf (HHUUKD)	RRID: CVCL_B3T9, https://hpscreg.eu/cellline/HHUUKDi009-A; PMID: 28132834
Human: HVRDi004-B (PGP1) iPSC	Harvard University (HVRD)	RRID: CVCL_F182; https://hpscreg.eu/cell-line/HVRDi004-B; PMID: 19911041
Software and algorithms		
Adobe Photoshop CS6	Adobe, Inc.	RRID: SCR_014199
Affinity Designer	Serif (Europe) Ltd.	RRID: SCR_016952
Excel 2021	Microsoft Office Professional Plus 2021	RRID: SCR_016137
EZ-C1 Silver version 3.91	Nikon	N/A
ImageJ	Schneider et al.[10]	RRID: SCR_003070; https://imagej.net/
NIS-Elements 4.51 Advanced Research	Nikon	RRID: SCR_014329
Origin Pro 2021	OriginLab Corporation	RRID: SCR_014212
Other		
6-well plates	Sarstedt	Cat#83.3920
CO_2 Incubator, CB170	Binder	Cat#9040-0163

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Injekt Solo 10 ml/ Luer	Braun	Cat#4606108V
Millicell Cell Culture Inserts	Millipore	Cat#PICM0RG50
Pasteur pipettes, glass	Brand	Cat#747715
Pasteur pipettes, plastic 3 ml	Heinz Herenz	Cat#1131303
Petri dish, 35 mm	Sarstedt	Cat#83.3900
Pipette tips MIKRO 0.1–10 µl graduated	Carl Roth	Cat#K138.1
Pipette tips UNIVERSAL 100–1200 µl	Carl Roth	Cat#2679.1
Qualitative filter paper	VWR	Cat#516-0813
Super glue gel	UHU	Cat#63261
Surface protector, Benchkote	Whatman	Cat#WHA2300916
Thermoblock, TB2; Alternative: Thermo Scientific™ Digital Dry Baths/Block Heaters	Biometra; Thermo Fisher Scientific	Discontinued; Cat#15362185
Tissue culture hood, HeraSafe	Thermo Fisher Scientific	Cat#51022515
Vibratome, Microm HM 650 V; Alternative: Vibratom 7000smz-2	Thermo Fisher Scientific; Campden Instruments	Discontinued; Cat#E7000smz-2
Wilkinson Classic Razor Blade	Wilkinson Sword	Cat#70517470

For reagents used for quality control and functional readout (steps 5–6), please refer to Petersilie et al.[1]

MATERIALS AND EQUIPMENT

Cortical Differentiation Medium IV, CDM IV (Day 70 on)]

Reagent	Final concentration	Amount
B-27 Supplement	2%	4 mL
Chemically Defined Lipid Concentrate	1%	2 mL
DMEM/F-12	1:1	168 mL
GlutaMAX Supplement	2 mM	2 mL
Heparin	5 µg/mL	100 µL of 10 mg/mL stock
Fetal Bovine Serum	10%	20 mL
N-2 Supplement	1%	2 mL
Penicillin/Streptomycin Solution	100 U/mL penicillin and 100 µg/mL streptomycin	2 mL
Total	**N/A**	**~200 mL**

Store at 4°C for up to 2 weeks.

Neural Differentiation Medium (NDM)

Reagent	Final concentration	Amount	DIV
Ascorbic Acid	200 uM	50 µL	22–45
B-27 Plus Supplement	2%	1 mL	46–70+
B-27 Supplement –Vit A	2%	1 mL	6–45
BDNF	20 ng/mL	100 µL	22–45
DHA	10 uM	16.7 µL	22–45
Dibutyryl cAMP	50 uM	25 µL	22–45
EGF	20 ng/mL	100 µL	6–21
FGF2	20 ng/mL	100 µL	6–21
GlutaMax	1%	500 uL	6–70+
MycoZap	0.2%	100 µL	6–70+
Neurobasal A	1:1	48 mL	6–70+
NT-3	20 ng/mL	100 µL	22–45
Pen/strep	1%	500 uL	6–70+
Total	**N/A**	**~50 mL**	**N/A**

Store at 4°C for up to 2 weeks.

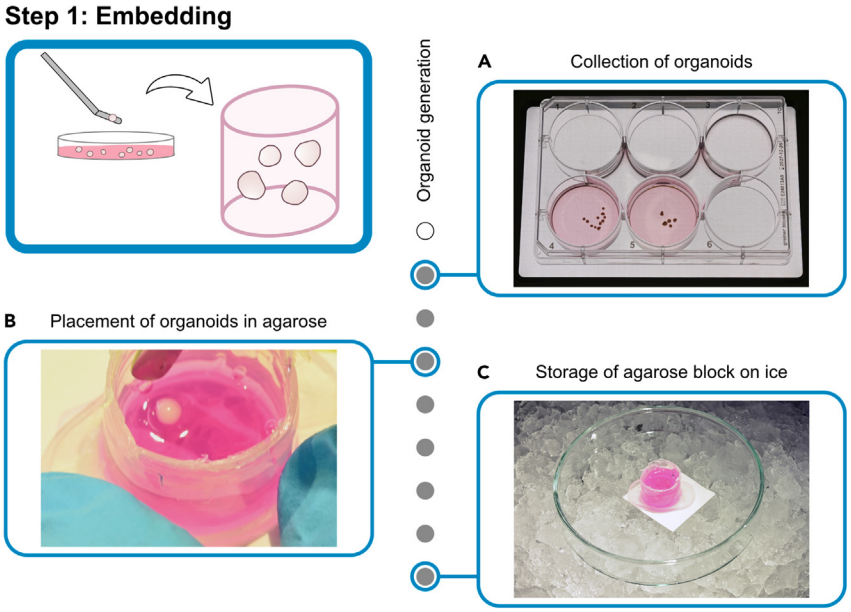

Figure 1. Details of the embedding process of cortical brain organoids in LMP agarose (step 1)
The first step in the generation of cultured cortical brain organoid slices (cBOS) is the embedding of the organoids. (A) Cortical brain organoids are stored in a medium-filled six-well plate. Organoids shown here are DIV 70 and DIV 78. (B) The image shows the placement of organoids in a custom-built mold filled with LMP agarose and the storage of the LMP agarose block on ice (C).

STEP-BY-STEP METHOD DETAILS

Step 1: Embedding

Timing: 15 min

This step involves the embedding process of cortical brain organoids in LMP agarose (see Figure 1).

1. Wash cortical organoids.
   a. Place cortical organoids (DIV 70–79, which are stored in medium) at your workstation (bench, if possible in sterile conditions).

   Note: Keep the temperature of the medium at ~36°C to ensure correct pH until embedding.

   Note: Penicillin-Streptomycin is included in the medium to avoid bacterial contamination.

   Note: Organoids too large and containing a necrotic core cannot be used to generate cBOS.

   b. Transfer each organoid from the medium into a 35 mm Petri dish filled with HBSS using a blunt-tip 3 mL Pasteur pipette.
   c. Carefully wash organoids by slowly pipetting them up and down in the pipette.
2. Place custom-build mold (cut upper end of a 10 mL syringe) onto a piece of absorbent filter paper.
3. Add LMP agarose to its bottom.

   Note: Avoid any air bubbles.

4. Carefully transfer a single organoid on a spatula (with blunt 3 mL Pasteur pipette).
5. Remove HBSS with filter paper.

⚠ CRITICAL: Do not touch the organoid with the filter paper. Troubleshooting 2.

6. Use spatula to place and arrange the organoid in agarose.

   Note: Excess liquid will result in improper embedding and the organoid will drop out of the agarose block during slicing process. Troubleshooting 2.

7. Repeat step 6 until you have several organoids placed in agarose (number depends on their size, here: up to five).

   Note: Try to place the organoids roughly in the same z-plane to speed up the slicing step.

8. Cover all organoids with LMP agarose.

   Note: Avoid any air bubbles.

9. Store mold in a glass petri dish on ice for a maximum of 10 min until the agarose solidifies. Troubleshooting 3.

   ⚠ CRITICAL: Try to work as quick as possible in this step.

Step 2: Slicing

Timing: 10 min (per agarose block)

This step details the slicing procedure of cortical brain organoids (see Figure 2).

10. Carefully remove solidified agarose block out of the mold.
11. Put glue onto a mounting plate.
12. Place agarose block in the glue.

    Note: Avoid any liquid or air bubbles under the agarose block. Troubleshooting 1.

13. Place mounting plate in ice-cold chamber, add the cooling element and ice-cold HBSS.

    Note: Depending on the handling and buffer system of your solution, be aware of the potential necessity to use carbogen to ensure correct pH.

14. Make 300 μm thick slices.

    Note: Adjust slice settings to ensure smooth cutting of the tissue. The velocity, angle of the blade etc. vary between specific slicers and blades used.

    Note: The time needed for slicing depends on the size of the organoids and mold and may vary between 5 and 15 min.

15. Collect slices with reverse end of a glass Pasteur pipette in Petri dish.

    Note: Quality control via visual inspection of each slice is important. Slices should be homogeneous in appearance with no necrotic regions. Areas of necrosis may be visible as tissue damage i.e. visible tissue rupture or lack of tissue in the inner part (see Figure 2D). Discard slices that are mechanically damaged or from the border region.

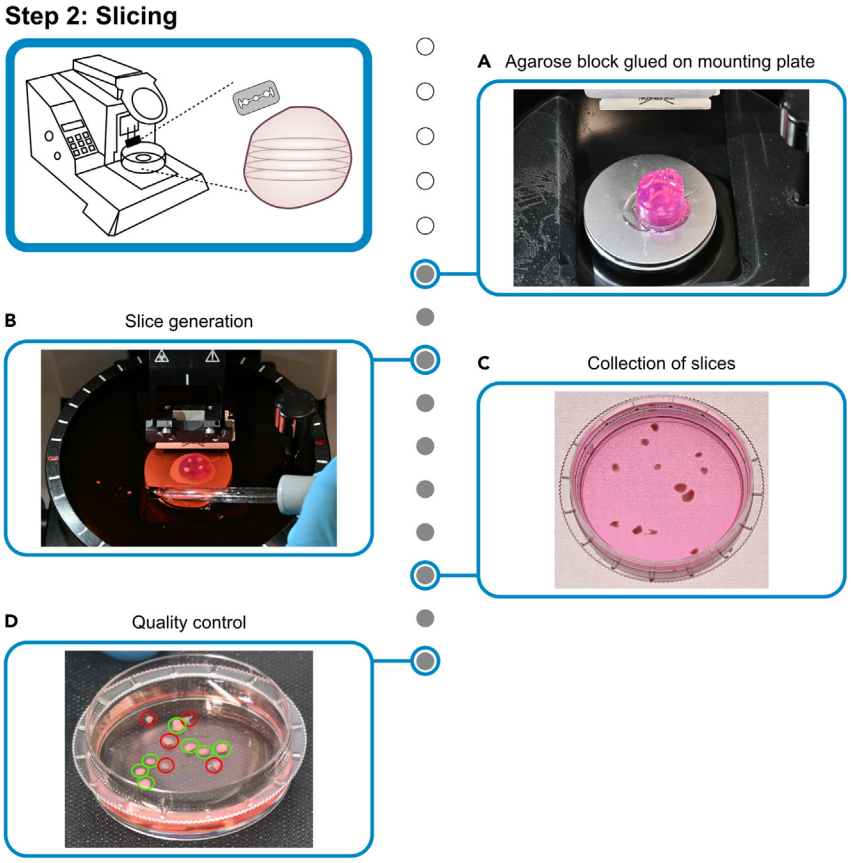

Figure 2. Details of the slicing process of cortical brain organoids in LMP agarose (step 2)
(A) Image shows the organoids embedded in a block of LMP agarose, glued on a mounting plate and placed in a vibratome bath.
(B) Illustrated are the slices floating in HBSS after the blade hit the LMP block.
(C) Finally, brain organoid slices are collected in a Petri dish.
(D) Slices used for cultivation (green circles) and those discarded (too small or ruptured, red circles) are indicated.

⚠ CRITICAL: Try to be as quick as possible in this step.

Step 3: Transfer

Timing: 10 min

This step involves how to get the slices sterile and how to transfer them to the culture inserts for the cultivation (see Figure 3).

16. Place Petri dish with slices in the sterile hood.
17. Take HBSS-filled Petri dishes out of the incubator.
18. Collect slices with reverse end of a sterile glass Pasteur pipette in Petri dish filled with pre-warmed HBSS (36°C). Repeat this step four times changing pipette and dish with every wash.

Note: This step ensures the sterility of the slices.

19. Take six-well plate with inserts out of the incubator.
20. Place up to five slices on culture inserts using a cut 1 mL tip. Troubleshooting 5.

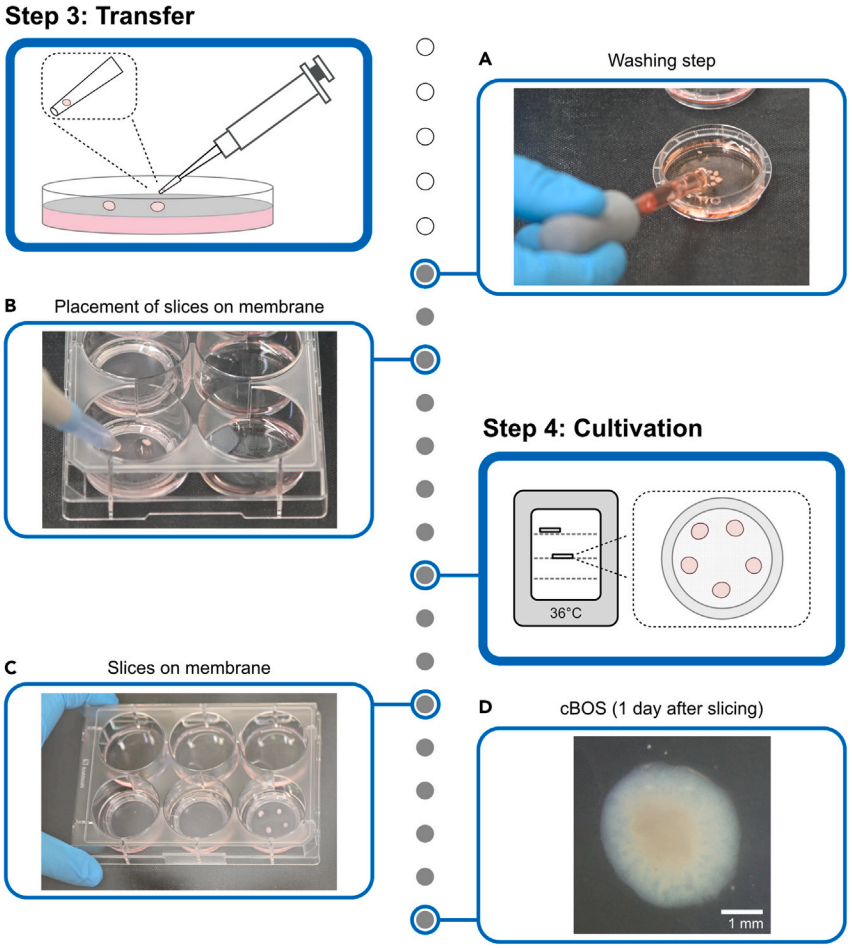

Figure 3. Details of the transfer of organoid slices on culture inserts and their cultivation (steps 3 and 4)
(A) Organoid slices are washed in HBSS for four times and (B) placed on membranes of culture inserts.
(C) Regularly four or up to five slices can be placed on one membrane.
(D) Representative image of a cBOS one day after slicing. Scale bar, 1 mm.

⚠ CRITICAL: Tip should be blunt and big enough to avoid tissue damage.

Note: Quality control via visual inspection of each slice is important. Slices should be homogeneous in appearance with no necrotic regions. Discard slices that are mechanically damaged.

21. Remove excess HBSS using a 10 μL tip on an evacuator.

⚠ CRITICAL: Do not touch the slices with the tip and do not get too close to them to prevent sucking them in. Troubleshooting 4.

22. Return the six-well plate to the incubator in a humidified atmosphere at 36°C and 5% CO_2 (static conditions, no orbital shaker).

Note: Use of an orbital shaker is disadvantageous because this will disturb adherence of cBOS to the membrane.

Step 4: Cultivation

Timing: 0–30 days

This step is a description of what to do during the cultivation of cBOS (see Figure 3).

23. Change the entire medium (750 μL) every day.

Note: Carefully place the pipette tip at the edge of the culture insert. Troubleshooting 4.

Note: After one day, slices should be already attached to the membrane.

⚠ CRITICAL: There should be no air bubbles in the medium. Also prevent air bubbles under the membrane.

Step 5: Quality control

This step is important after the cultivation period to control for the quality and viability of your cBOS and determine cell types. We ensured the absence of a necrotic core using PI staining (see Figure 4A). The presence of mature glial cells and neurons was determined using fluorescence-based using immunohistochemistry (Figure 4B). A detailed protocol for both procedures can be found in Petersilie et al.[1]

Step 6: Functional readout

Timing: 10–24 days

Timing: 10 min per well (for steps 24–27)

Timing: 1 h per cBOS (for steps 28–32)

This step addresses the functionality of cBOS and their use for different physiological experiments. Here, we describe the procedures for and show results obtained upon expression of a genetically encoded sensor for intracellular ATP in cBOS neurons and the changes in neuronal ATP determined following transient metabolic inhibition (see Figure 5). The FRET-based sensor ATeam1.03^{YEMK} (ATeam) is expressed under the human synapsin 1 promoter. For further details of the viral transduction see Petersilie et al. (2024).[1]

24. Dilute the adeno-associated virus (AAV) 1:1 (virus titer: 3.1×10^{12} genome/mL) in Dulbecco's Phosphate Buffered Saline (dPBS).
25. Take a six-well plate with inserts and cBOS out of the incubator.
26. Apply 1 μL of the AAV-solution on top of each cBOS (DIV94-101).
27. Return the six-well plate to the incubator in a humidified atmosphere at 36°C and 5% CO_2 (static conditions) and keep cBOS for additional 10–24 days in the incubator.

The following steps detail the procedures for imaging of intracellular ATP, see Petersilie et al. (2024).[1]

28. Place insert with cBOS in a 30 mm Petri dish filled with medium (here: CDM IV or NDM).
29. Cut out a single transduced cBOS using a sterile scalpel.
30. Transfer cBOS to a fluorescence imaging system (e.g., upright microscope equipped with a 40x water immersion objective, a camera and a monochromator or LED light source).
31. Fix cBOS with a grid, place it in a recording chamber and continuously perfuse it with saline at 37 ± 1°C.

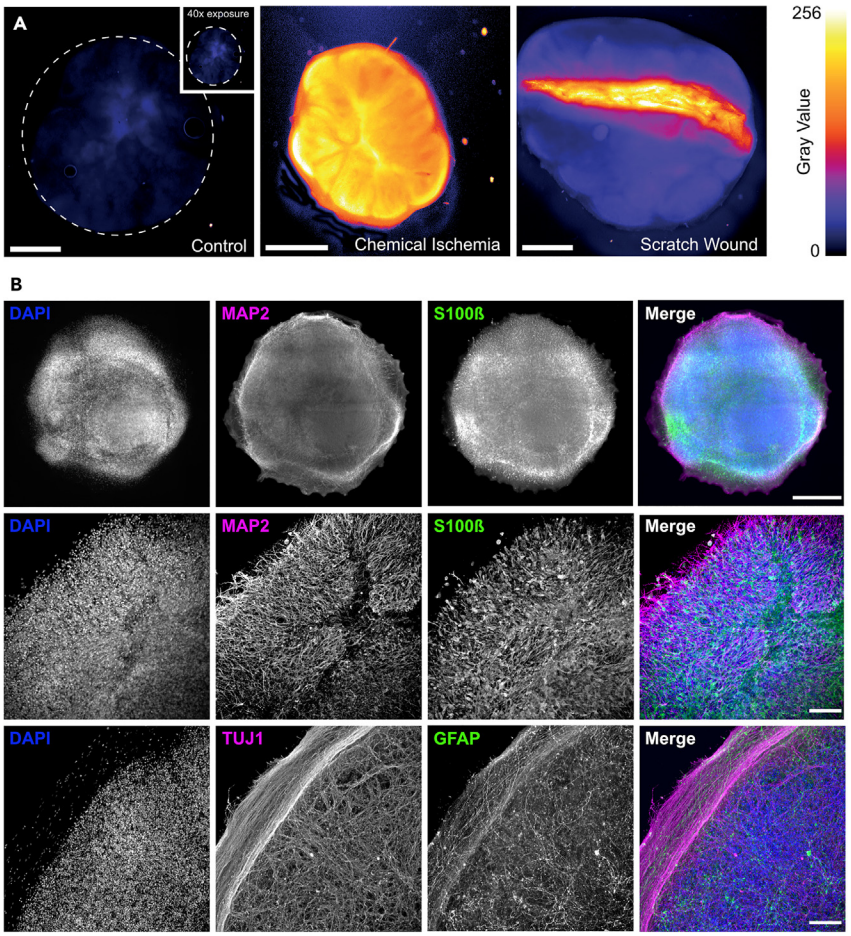

Figure 4. Cellular integrity and composition of cBOS (step 5)

(A) Images of propidium iodide- (PI-) stained cBOS at DIV 116. **(Left)** Virtually no PI label is found in untreated cBOS (Control), indicating their cellular integrity and low level of cell death. The inset shows the same tissue exposed 40 times longer for documentation purposes. The dotted lines indicate the outer limits of the cBOS. **(Middle)** PI-labeled cBOS after a 10 min exposure to metabolic inhibitors to induce transient chemical ischemia. The entire cBOS is highly labeled, indicating that the vast majority of cells are severely damaged. (Right) After mechanical tissue damage by a scratch wound, cBOS show strong PI labeling along the wound, while the rest of the tissue remains PI negative. All experimental manipulations were performed in parallel with untreated controls on sibling cBOS using the same procedures. The intense labeling in the treated cBOS indicates that the labeling process was successful and that the absence of labeling in the native control tissue is correct. The linear color look-up table (far right) shows the intensity of the label. White (gray value 256) = highest intensity; black (gray value 0) = no label. Scale bars: 2 mm.

(B) Immunohistochemical labeling for astroglial (GFAP and S100β) and neuronal (TUJ1 and MAP2) marker proteins. **Upper panel:** Stitched wide field images display the overall structure and size of triple-labeled cBOS (DAPI, S100β and MAP2) at DIV 108. Scale bar, 500 μm. Middle panel: A representative extended-focus projection showing the detailed cellular organization of MAP2- and S100ß-positive cells in cBOS at DIV 108. In addition, the DAPI label and the merge of all three channels is displayed. Scale bar, 100 μm. **Lower Panel:** Representative extended-focus projection displaying details of GFAP-positive cells and TUJ1-positive cells in cBOS at DIV 116. In addition, the DAPI label and the merge of all three channels are shown. Scale bar, 100 μm.

32. Excite ATeam at 434 nm and split emission at 500 nm by an image splitter. Emission channels should be band-pass filtered (483/32, eCFP donor; 542/27, mVenus acceptor).

EXPECTED OUTCOMES

Depending on the size of the organoids used for preparation of cBOS, the protocols described above enable the generation of about 3–4 cBOS per individual organoid (Figure 2). The use of

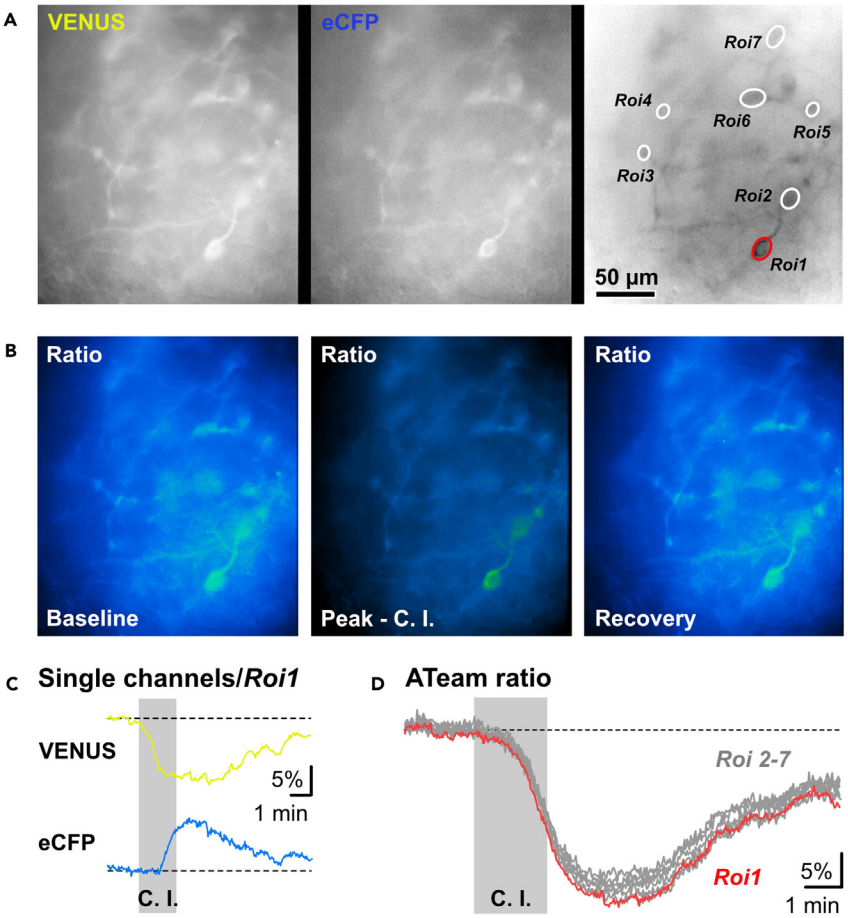

Figure 5. Expression of a genetically-encoded nanosensor enables dynamic imaging of changes in intracellular ATP in cBOS (step 6)

(A) Wide field images of fluorescence emission (left: Venus/527 nm; center: eCFP/475 nm) of neurons in a cBOS at DIV 118, expressing the FRET-based nanosensor ATeam1.03^{YEMK}. Right: Inverted image to illustrate regions of interest (Roi 1–7) from which measurements shown in D were taken from. Scale bar, 50 µm.

(B) Left: Wide field image of the neuronal ATeam1.03^{YEMK} ratio (VENUS/eCFP) during baseline conditions. Center: Image of the ATeam1.03^{YEMK} ratio recorded at the peak of its decline induced by chemical ischemia for 2 min ("C.I."). Left: Image of the ATeam1.03^{YEMK} ratio recorded about 10 min after C.I., illustrating partial recovery. Images were processed with customized look up table (LUT) for visualization purposes.

(C) Relative changes in the fluorescence emission of VENUS (top, yellow trace) and eCFP (bottom, blue trace) channels recorded in Roi1 and induced by C.I. for 2 min (indicated by the gray area).

(D) Relative changes in the ATeam1.03^{YEMK} ratio of 7 individual neurons (Roi1-7) induced by C.I. for 2 min (gray area). The red trace highlights Roi1, Roi2-7 are depicted in gray. Images in (A) and (B) are average-intensity-projections out of z-stacks.

cBOS therefore increases overall preparation yield by allowing multiple slices to be generated from each brain organoid. This also improves standardization in studies where the effects of drugs are being compared. At around 30 days after slicing, individual cBOS display a circular homogeneous shape and a diameter of around 2–3 mm, which further increases with prolonged culturing (Figures 3, 4A, and 4B). No repeated slicing is necessary because cBOS do not significantly increase in thickness, but rather in diameter. To ensure the absence of necrotic regions in cBOS, staining with Propidium Iodide (PI), which is an established marker for cell death, can be performed as described by.[1] In normal, undisturbed cBOS, PI staining should essentially be absent, revealing negligible cell death as illustrated in Figure 4A. As positive controls for the PI staining, cBOS can e.g., be transiently (here: 10 min) exposed to blockers of cellular ATP production to mimic ischemic conditions in the brain, a protocol called "chemical ischemia."[11] An alternative procedure is to perform a mechanical

injury by a scratch using a sharp needle or scalpel. Both manipulations should result in bright PI staining, in the first case of the entire cBOS, in the second case of cells close to the injured tissue (Figure 4A).

Besides negligible cell death, a further expected outcome is that cBOS maintained in culture for at least 30 days contain both mature neurons and astrocytes and form functionally-active neural networks based on the presence of mature neurons and glial cells. The identity of cells can be probed for by performing immunohistochemistry with established markers for neuronal structures (e.g., MAP2 and/or TUJ1) or for cells of the astrocyte lineage (e.g., S100ß and/or GFAP). As shown in Figure 4B, such stainings reveal dense MAP2-positive labels of cells reminiscent for neurons, suggesting mature neuronal structures in cBOS. TUJ1 and MAP2 labeling in addition indicates fasciculation and formation of neuronal fiber tracts. Such fiber tracts are crucial for the formation of minimal neuronal networks. Furthermore, cBOS are expected to show cells positive for S100ß- and GFAP, revealing the presence of mature astrocytes as well as of cells of the astrocyte lineage (Figure 4B).

Owing to their restricted thickness, cBOS provide optimal access to neural cells that can be directly probed with respect to their functionality. Functional approaches include the extracellular measurement of electrical activity by multi-electrode recordings (MEAs) or the recording of the electrophysiological properties and signals of individual cells by whole-cell patch-clamp as shown recently.[1] The latter technique enables an in-depth investigation of synaptic properties and synaptic transmission under well-controllable experimental conditions in these developing human neural networks. The good experimental accessibility also allows imaging of intracellular ion transients including the analysis of single cell or network calcium signaling employing chemical indicator dyes and standard approaches available to many laboratories like confocal or wide field imaging in cBOS.[1,4]

Another advantage of cBOS is the relatively easy and simple experimental use of genetically encoded probes that can be expressed in defined cell types using specific promoters. These e.g., include well-established sensors for the measurement of intracellular calcium or of cellular metabolites. Figures 5A and 5B illustrate the expression of the genetically encoded nanoprobe ATeam^{YEMK} in neurons employing a single-stranded adeno-associated-vector controlled by the neuron-specific human synapsin 1 promoter[12] and visualized using a standard wide-field imaging system.[13] This sensor allows the FRET-based imaging of changes in intracellular ATP such as those induced by a brief period of metabolic inhibition ("chemical ischemia", here: 2 min) in cBOS (Figures 5C and 5D).[1] Altogether, this demonstrates that cBOS will be widely applicable to different laboratories and have a wide range of potential applications enabling the morphological and functional assessment of developing human neural networks.

LIMITATIONS

A main limitation of cBOS is the limited time they can be kept in culture. Even prolonged culturing periods of about a year (see Petersilie et al., 2024) are rather brief when compared to the development and maturation of the human brain, which spans over more than 20 years. Moreover, cBOS prepared according to the presented protocol lack cells of mesodermal origin including microglia. The latter are known to play important roles in synaptic pruning and the development of neuronal networks. Furthermore, cBOS do not contain vascularization, and therefore not only lack blood flow, but also lack the influence of vasculature on the maturation of neurons and neuronal networks. Last, but not least, one of the advantages of cBOS, the reduction of tissue complexity, is also a disadvantage as they are less comparable to the developing human brain than brain organoids.

TROUBLESHOOTING

Problem 1

The entire LMP agarose block containing the cortical organoids moves or dissolves when the blade touches the block (related to step 2, point 12).

Potential solution

The glue was already too dry and/or there was too much liquid under the block. Try to be quick when putting the glue on the mounting plate or use a filter paper to carefully remove excess liquid if necessary.

Problem 2

The cortical brain organoids fall out of the LMP agarose block during the slicing procedure (related to step 1, point 5 & 6).

Potential solution

There was too much HBSS or air bubbles around the organoids when they were covered with LMP agarose. A cover of liquid or air around the organoids is the result. Try to transfer the organoids with as little liquid around them as possible and carefully destroy air bubbles with a cannula if necessary.

Problem 3

cBOS are stuck to the LMP agarose slices and disrupt when sucking them up to the transfer them (related to step 1, point 9).

Potential solution

The agarose block has been stored on ice for too long. The process of embedding and slicing should be as quick as possible. Usually the slices will dissolve out by themselves or by gentle suction with the glass Pasteur pipette.

Problem 4

The cBOS do not attach to the membrane of the culture insert (related to step 3, point 21 & step 4, point 23).

Potential solution

There was too much liquid around the slices. Remove any liquid on the membrane after the transfer and avoid swapping media on the membrane during the media change.

Problem 5

cBOS stick to each other or to the edges of the culture insert during the cultivation (related to step 3, point 20).

Potential solution

cBOS were placed too close to each other or to the edges of the culture insert. Leave as much space as possible between the slices and the edges, as the slices will grow during the cultivation and may need to be cut out depending on the assay.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Christine R. Rose (rose@hhu.de).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to and will be answered by the technical contact, Laura Petersilie (Laura.Petersilie@hhu.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.R.R., K.W.K., and L.P.; methodology, L.P., S.L., S.H., and K.W.K.; formal analysis, L.P., K.W.K., and L.A.N.; investigation, L.P., S.H., K.W.K., and L.A.N.; writing – original draft, L.P., K.W.K., and C.R.R.; writing – review and editing, all authors; funding acquisition, C.R.R. and A.P.; supervision, C.R.R. and A.P. All co-authors approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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